# Cross-Linking of CD53 Promotes Activation of Resting Human B Lymphocytes

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The CD53 pan-leukocyte glycoprotein is a member of the recently described tetraspan family of cell membrane proteins. The structure and functional characteristics of these molecules indicate that they may play important roles in transmembrane signaling in different cells. Recently, it was reported that cross-linking of CD53 on human B cells led to an increase in cytoplasmic calcium fluxes. In the present study, we wished to further explore the possible role of CD53 in functional B cell responses. Cross-linking of CD53 with the use of the mAb MEM-53 and a polyclonal sheep anti-mouse Ig promoted activation of resting B cells into the G1 phase of the cell cycle as judged by increased expression of the early activation Ag CD69, increases in cellular volume, RNA synthesis, and c-myc protein levels, and enhanced binding of 7-aminoactinomycin D. In contrast, MEM-53 alone had no detectable effects. Cross-linking of anti-CD53 induced negligible S phase entry in the absence of other stimuli. However, cytokines, in particular IL-2 and IL-4, potentiated the DNA synthesis induced by cross-linking of CD53. Furthermore, cross-linking of the CD53 Ag induced Ig production in the presence of T cell supernatant. Taken together, the data suggest that CD53 plays an important functional role in B cell activation and differentiation. *The Journal of Immunology,* 1994, 153: 4997.

Abs directed against sIg[2] can mimic binding of soluble Ag to the B cell receptor and thus trigger B cells to enter the cell cycle (1, 2). In addition, Abs against several other B cell surface Ags have been shown to influence B cell activation and/or proliferation alone or in the presence of exogenous lymphokines such as IL-2, IL-4, and IFN-γ (3–5), indicating that several Ags on B cells can elicit functional responses.

The CD53 Ag is a 32- to 42-kDa glycoprotein that is broadly expressed on leukocytes, including B cells and T cells, but is only present on a subset of thymocytes (6). CD53 is homologous to the rat OX-44 Ag (7) and belongs to the recently described tetraspan family of membrane proteins (8). The other members of the tetraspan family are the leukocyte cell surface molecules CD9 (9, 10), CD37 (11, 12), CD63 (13, 14), R2 (CD82; 15, 16), and TAPA-1 (CD81) (17); the tumor-associated Ags CO029 (18) and L6 (19); the mink Ag TI1 (20); and the schistosomal Ag Sm23 (21). The predicted structure of this superfamily, as derived from hydropathy analyses of the deduced protein sequence (14, 21, 22), biochemical analyses of in vitro-translated TAPA-1 (17), and epitope mapping of anti-rat CD53 mAb (23), suggests a topology in which the N- and C-termini of the proteins remain intracellular, whereas the major hydrophilic domain, located between transmembrane domains 3 and 4, is extracellular. Several lines of evidence suggest that the CD53/OX-44 Ag can play an important functional role. Thus, it has been shown that mAbs to OX-44 stimulate the phosphatidylinositol signaling pathway and induce the appearance of tyrosine phosphorylated proteins in the rat leukemia cell line RNK-16. Furthermore, one mAb against the OX-44 molecule (7D2) is mitogenic for splenic T cells and augments TCR-mediated proliferation (24). Moreover, anti-OX-44 Abs have been shown to induce release of nitric oxide in rat macrophages (25). In humans it has recently been shown that the MEM-53 mAb, directed against the CD53 Ag, induced

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[2] Abbreviations used in this paper: sIg, surface Ig; AFP, α-fetoprotein; SAM, surface sheep anti-mouse Ig; BrdU, bromodeoxyuridine; SAC, *Staphylococcus aureus* bacteria; 7-AMD, 7-aminoactinomycin D.



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cytoplasmic calcium fluxes in B cells, monocytes, and granulocytes (26).

In the present report, we have investigated further the functional role of CD53 in B cells. Our data demonstrate that signaling through the CD53 Ag induces B cell activation and differentiation in a manner similar to signaling through sIg.

## Materials and Methods

### *Abs and reagents*

The mAb against CD53 (MEM-53) was described earlier (27). Biotinylated anti-CD69 (FN61), anti-AFP (K-57), anti-human IgG, and anti-human IgM were generated in our laboratory (28). Rabbit anti-human $F(ab')_2$ fragments of IgG were made in our laboratory. Polyclonal anti-$\mu$ Ab ($F(ab')_2$ fragments) and anti-CD71-FITC and irrelevant IgG1-FITC conjugates were obtained from Dakopatts (Copenhagen, Denmark). All mAbs were used at predetermined optimal concentrations unless otherwise stated. $\gamma$-Globulin was obtained from Kabi Vitrum AB (Stockholm, Sweden). Streptavidin-alkaline-phosphatase, *p*-nitrophenylphosphate substrate, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Streptavidin-phycoerythrin was purchased from Vector Laboratories (Burlingame, CA). Dynabeads M-450 coated with anti-CD19 mAb (Dynabeads M-450 Pan B, product 11104), rat anti-mouse IgG1 (product 110.12), and SAM (DETACHaBEAD, product 125.01/02) were obtained from Dynal (Oslo, Norway). $F(ab')_2$ fragments of sheep anti-mouse Ig (H + L chain) were provided from Jackson Immunoresearch Laboratories (West Grove, PA). rIL-2, rTNF-$\alpha$, rTNF-$\beta$, and rIFN-$\gamma$ were kindly provided by Hoffmann LaRoche (Basel, Switzerland) and rIL-4 from Schering Corp. (Bloomfield, NJ). BrdU was obtained from Sigma, Hoechst 33258 was purchased from Riedel de Haen (Seelze, Germany), and propidium iodide was obtained from Calbiochem Corp. (La Jolla, CA). Fixed SAC and 7-AMD were obtained from Calbiochem-Behring (Cambridge, UK). PHA was purchased from Wellcome Research Laboratories (Beckenham, UK).

### *Preparation of $F(ab')_2$ fragments of IgG mAb*

IgG1 of anti-CD53 and anti-AFP were purified on protein A-Sepharose 4 fast flow column (Pharmacia, Stockholm, Sweden). $F(ab')_2$ fragments were prepared as described by Parham (29). The purity of $F(ab')_2$ fragments was demonstrated by measuring a >99% reduction in cell staining with FITC-conjugated Abs to the Fc part of mouse IgG, compared with the intact IgG mAb (data not shown).

### *Isolation of B lymphocytes*

B lymphocytes were positively selected from whole blood of healthy donors by using Dynabeads M-450 coated with anti-CD19 mAb (Dynabeads M-450 Pan B) followed by detachment of beads with DETACHaBEAD (Dynal) as described previously (30). Briefly, buffy coats (50 ml) were mixed with 0.01 M EDTA in 25 ml RPMI 1640. Dynabeads M-450 Pan B were added to the cell suspension with a target-to-bead ratio of 1:10. The mixture was incubated for 30 min at 4°C, and rosetted cells were washed six times in RPMI 1640 with 1% FCS and resuspended in 100 $\mu$l of the same medium before adding 100 $\mu$l DETACHaBEAD. Detachment was performed under gentle rotation at ambient temperature for 45 min. To obtain detached cells, the tube was placed on a magnet to remove beads. Isolated B cells were washed twice in RPMI 1640 with 1% FCS before they were used in functional assays.

### *Production of T cell supernatant*

Mononuclear cells were isolated from five healthy donors by gradient centrifugation, as described by Bøyum et al. (31). Cells were pooled, diluted to $2 \times 10^6$/ml in RPMI with 5% FCS, and stimulated with PHA at a concentration of 1 $\mu$g/ml for 24 h. Cells were removed by centrifugation at 400 $\times$ *g* for 10 min and the supernatant was collected and filtered through a 0.22-$\mu$m filter. The T cell supernatant was tested for synergistic activity on anti-$\mu$-stimulated B cells and used at a final concentration of 5% (v/v).

### *Cell culture*

Isolated B cells were cultured in triplicate wells in 96 U-bottom microplates in RPMI 1640 with 1% FCS ($7.5 \times 10^4$ cells/0.2 ml). Cells were incubated with anti-CD53 mAb (MEM 53) at a final concentration of 5 $\mu$g/ml for 1 h at 4°C, followed by two washes. SAM was added at a concentration of 10 $\mu$g/ml and was present for the whole stimulation period. In control experiments, B cells were stimulated with anti-$\mu$ at a final concentration of 35 $\mu$g/ml for 3 days. Recombinant cytokines such as IL-2 (20 U/ml), IL-4 (40 ng/ml), IFN-$\gamma$ (20 ng/ml), TNF-$\alpha$ (20 ng/ml), TNF-$\beta$ (20 ng/ml), or T cell supernatant (5%) were added to the cultures at the start of stimulation.

### *RNA and DNA synthesis*

B cells were cultured in triplicate wells in 96 U-bottom microplates as described above. To determine RNA synthesis, cells were stimulated for 30 h, and 0.5 $\mu$Ci [$^3$H]uridine was added to the cultures for the last 10 h. To determine DNA synthesis, cells were stimulated for 72 h and 0.5 $\mu$Ci [$^3$H]thymidine was added to the cultures for the last 24 h. Each value represents the mean of triplicate cultures.

### *Cell volume determinations*

Isolated B cells were analyzed for initial volume distribution as well as volume increase upon stimulation for 20 h with anti-CD53 cross-linked with SAM or with anti-$\mu$. Cells were assayed on a modified Coulter counter as described elsewhere (32). The volume distribution data were based on counts of 20,000 cells.

### *Isolation of CD69$^-$ B cells*

Positively isolated B cells ($20 \times 10^6$/ml) were rosetted with rat anti-mouse IgG1 Dynabeads coated with anti-CD69 (FN61) at a target-to-cell ratio of 1:10 for 30 min at 4°C. Rosetted CD69$^+$ B cells were removed with a magnet and the CD69$^-$ B cell population was used in activation assays after being tested in flow cytometry for CD69 expression.

### *Determination of activation Ag expression*

CD69$^-$ B cells ($10^6$) in 1 ml of RPMI 1640 with 1% FCS were incubated alone or with anti-CD53/SAM, anti-AFP/SAM, or with polyclonal anti-$\mu$ at concentrations as described above. Stimulation was performed for 4 h and 20 h to estimate CD69 expression and for 48 h to determine CD71 expression. After washing, cells were incubated with 100 $\mu$g of irrelevant mAb (anti-AFP) for 30 min to saturate SAM binding capacity. Cells were stained with biotinylated anti-CD69 (FN61), followed by a strepavidin-phycoerythrin conjugate (Vector). To estimate transferrin receptor expression, cells were stained with an anti-CD71-FITC conjugate (Dakopatts). As control, cells were stained with biotinylated anti-AFP and streptavidin-phycoerythrin or with irrelevant IgG1-FITC. Flow cytometry was performed with a Becton Dickinson FACScan with logarithmic fluorescence scales. The data calculations are based on measurements of 10,000 cells.

### *7-AMD staining*

For the determination of 7-AMD binding, $10^6$ B cells were stimulated for 48 h with anti-CD53 cross-linked with SAM or anti-$\mu$, as described above, in the presence of T supernatant. 7-AMD staining was determined as described by Stokke et al. (33). Briefly, cells were fixed in 3% fresh paraformaldehyde for 24 h at 4°C. Fixed cells were washed once in PBS with 0.1% Triton X-100 (Sigma) and resuspended in the same buffer with 25 $\mu$g/ml 7-AMD (Calbiochem-Behring). Relative fluorescence intensity was measured on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

### *Cell cycle analysis*

To distinguish cells in the first and second cell cycle and determine the cell cycle distribution, $2 \times 10^6$ B cells in 1.5 ml RPMI 1640 plus 1% FCS were stimulated with either anti-CD53 cross-linked with SAM or anti-$\mu$ in the presence of T cell supernatant. BrdU (30 $\mu$g/ml; Sigma) was added

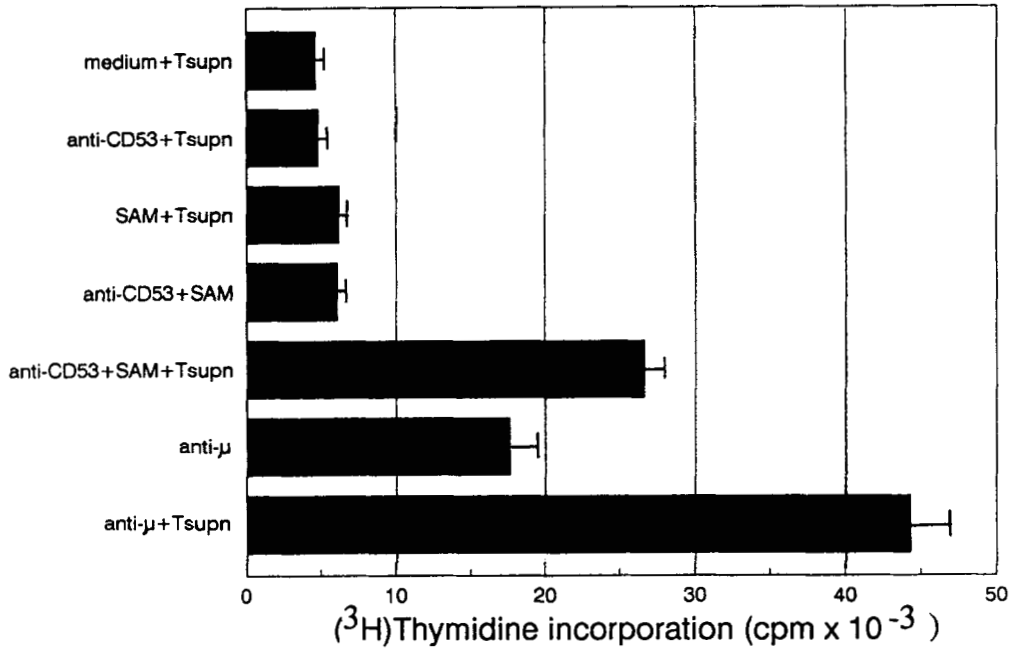


**FIGURE 1.** Cross-linking of the CD53 Ag induces B cell proliferation in the presence of T cell supernatant. B cells were stimulated with anti-CD53 in the presence or absence of SAM and T cell supernatant or with anti-$\mu$ as described in *Materials and Methods*. Cell proliferation was determined by [$^3$H]thymidine incorporation on day 3. Results are expressed as mean cpm ± SEM from 17 experiments.

after 24 h of stimulation (33). As control, B cells were stimulated with SAC and anti-$\mu$. After 48 h and 72 h of culture the cells were fixed in 100% methanol and stored at −20°C. Fixed cells were washed once in 0.15 M NaCl, 0.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), and 0.1% Triton X-100 and resuspended in the same buffer containing 1.5 $\mu$g/ml Hoechst 33258 (Riedel de Haen) and 1.0 $\mu$g/ml propidium iodide (Calbiochem). The samples were analyzed in a FACStar$^{Plus}$ flow cytometer (Becton Dickinson) equipped with two lasers tuned to 488 nm (200 mW) and UV (50 mW) for excitation of propidium iodide/light scatter (propidium iodide emission, >650 nm) and Hoechst 33258 (emission, 400 to 450 nm). The data were gated on propidium iodide fluorescence area vs pulse width to exclude aggregates. Analysis of the cell cycle distribution (propidium iodide fluorescence area) of the cells in the first and second cell cycle was done with the Modfit program (Verity Software House, Topsham, ME) after additional gating on cells with high and low Hoechst 33258 fluorescence, respectively.

### *B cell differentiation*

For the determination of Ig production, $0.5 \times 10^6$ B cells were incubated with 2.5 $\mu$g of anti-CD53 or irrelevant mAb (anti-AFP) for 1 h before the washing of cells and addition of SAM (10 $\mu$g/ml) in a total volume of 0.5 ml RPMI 1640 with 5% FCS. B cells stimulated with SAC at a final dilution of 1:40,000 were used as positive control. All wells contained 5% T cell supernatant or rIL-4 (40 ng/ml) during the whole stimulation period. B cells were stimulated for 6 days before washing twice in RPMI 1640 with 5% FCS. Cells were resuspended in the same media with 5% T cell supernatant or rIL-4 and were incubated for 24 h before supernatants were collected. Ig levels were determined by using a modified ELISA technique (34). ELISA (96-well) plates were coated overnight with 1 $\mu$g/ml (100 $\mu$l/well) of a rabbit anti-human $F(ab')_2$ antiserum. The plates were washed four times before cell supernatants were added (diluted 1:5 in PBS, 0.05% Tween 20, and 0.5% BSA). Human IgG (Kabi Vitrum AB) and IgM (isolated from a myeloma patient) were used to make standard curves. Incubation was performed for 2 h at 37°C followed by washing. Biotinylated mAbs against human IgG and IgM were added at optimal concentrations for 2 h followed by streptavidin-alkaline-phosphatase (Sigma) at a dilution of 1:2000. As a detection system we have used the alkaline phosphatate substrate *p*-nitrophenylphosphate (Sigma) diluted to 1 mg/ml in 1 M diethanolamine, pH 9.8, and staining was measured at 405 nm with an ELISA reader (Titertek Multiskan Plus).

## Results

### *The effect of cross-linking of the CD53 Ag on B cell proliferation*

We have performed cross-linking experiments with anti-CD53 and SAM and compared the responses with anti-$\mu$ stimulation of B cells. When anti-CD53 was cross-linked with SAM in the presence of T cell supernatant, a marked increase in the [$^3$H]thymidine incorporation was observed (Fig. 1). No effect of soluble anti-CD53 was noted on [$^3$H]thymidine uptake in the presence or absence of T cell supernatant. Furthermore, cross-linking of the CD53 Ag in the absence of T cell supernatant did not lead to induction of DNA synthesis. This indicates that cross-linking of the Ag was necessary for cell activation. As a negative control, we used a mAb directed against AFP (K-57) that shows no reactivity with B cells. K-57 did not induce increased [$^3$H]thymidine incorporation in the absence or presence of T cell supernatant even under cross-linking conditions (data not shown). When comparing the CD53 response with signaling through sIgM, we found that cross-linking of CD53 in the presence of T cell supernatant led to a [$^3$H]thymidine incorporation that was 54 ± 5.4% (mean ± SEM, $n$ = 17) of the response induced by

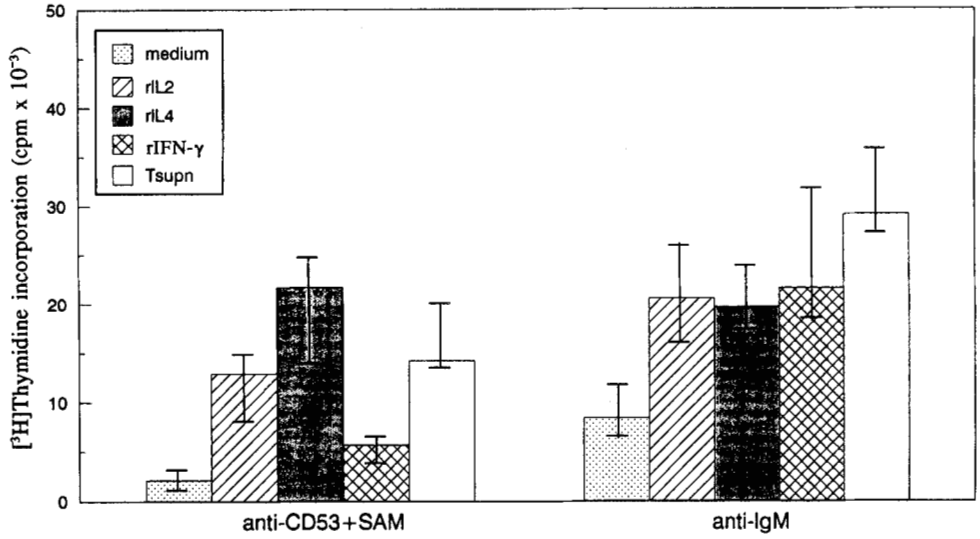


**FIGURE 2.** The effect of recombinant cytokines on CD53-induced B cell proliferation. B cells were stimulated with anti-CD53/SAM or anti-$\mu$ in the presence of recombinant cytokines or T cell supernatant as described in *Materials and Methods*. After 3 days, cell proliferation was determined by [$^3$H]thymidine incorporation. The background level of cytokines and SAM have been subtracted. Results are expressed as median ± range of four experiments.

anti-$\mu$ and T cell supernatant. The observed responses induced by Ab cross-linking were not due to Fc-$\gamma$R interactions as purified F(ab')$_2$ fragments of both anti-CD53 and control mAb were used in all experiments.

### *Cytokines potentiate the CD53-induced proliferation*

We next wanted to explore whether recombinant cytokines could interact with anti-CD53 to induce B cell proliferation. As shown in Figure 2, rIL-4 induced a 10-fold increase in the proliferative response of B cells activated through the CD53 Ag, whereas rIL-2 showed a weaker effect. Furthermore, rIFN-$\gamma$ did not potentiate the CD53-induced proliferation. This was in contrast to anti-$\mu$ activated B cells, in which rIL-2, rIL-4, and rIFN-$\gamma$ all showed a potentiating effect. Under these conditions, neither rTNF-$\alpha$ nor rTNF-$\beta$ was able to synergize with anti-CD53 or anti-$\mu$ in maintaining B cell proliferation (data not shown).

### *Cross-linking of CD53 promotes G1 entry of resting B cells*

Activation of cells from $G_0$ into the $G_1$ phase is accompanied by increases in cellular volume, activation Ag expression, gene expression, and RNA synthesis (35, 36). To address the ability of CD53 cross-linking to induce $G_1$ entry, we first determined RNA synthesis after 30 h of stimulation (37). As can be seen in Figure 3, cross-linking of anti-CD53 in the absence of T cell supernatant led to a marked increase in [$^3$H]uridine incorporation that was comparable to an anti-$\mu$ response. Furthermore, soluble anti-CD53 alone did not induce increased [$^3$H]uridine relative to the controls receiving medium only or SAM, both in the absence and presence of T cell supernatant. The CD69 Ag is an early activation marker that can be detected on B cells as early as 3 to 4 h after stimulation with anti-$\mu$ (38). Addition of the CD53 mAb alone did not induce CD69 expression on CD69$^-$ B cells (data not shown). However, by cross-linking anti-CD53 with SAM for 4 h we observed a marked increase in CD69 expression. As can be seen in Figure 4*A*, the CD69 expression induced by CD53 cross-linking was even higher than the expression induced by anti-$\mu$, indicating that activation of B cells through the CD53 Ag promotes a strong competence signal to B cells. This was also confirmed by Western blot analysis of c-Myc protein at 4 h of stimulation. The Myc protein level was somewhat higher in B cells stimulated with anti-CD53/SAM compared with anti-$\mu$-stimulated cells (data not shown). The CD69 expression and c-Myc protein levels measured at 4 h represent early $G_1$ activation parameters, whereas the CD69 expression and cellular volume distribution measured after 20 h stimulation represent intermediate (mid $G_1$) activation parameters. After 20 h of stimulation, cross-linking of CD53 induced CD69 expression comparable with that of anti-$\mu$-stimulated cells (Fig. 4*B*). It is well established that activation of lymphocytes from a resting state into the $G_1$ phase of the cell cycle is accompanied by a successive increase in cellular volume (32). As can be seen in Figure 5, cross-linking of CD53 for 20 h induced an increase in cellular volume comparable with that induced by anti-$\mu$.

Actinomycin D is known to bind to DNA but shows a stronger binding to DNA in open than condensed chromatin, hence the 7-AMD binding increases upon cell activation. Furthermore, 7-AMD binding has been shown to correlate with DNase 1 susceptibility and RNA content in lymphocytes and hence can be used as a parameter of cell

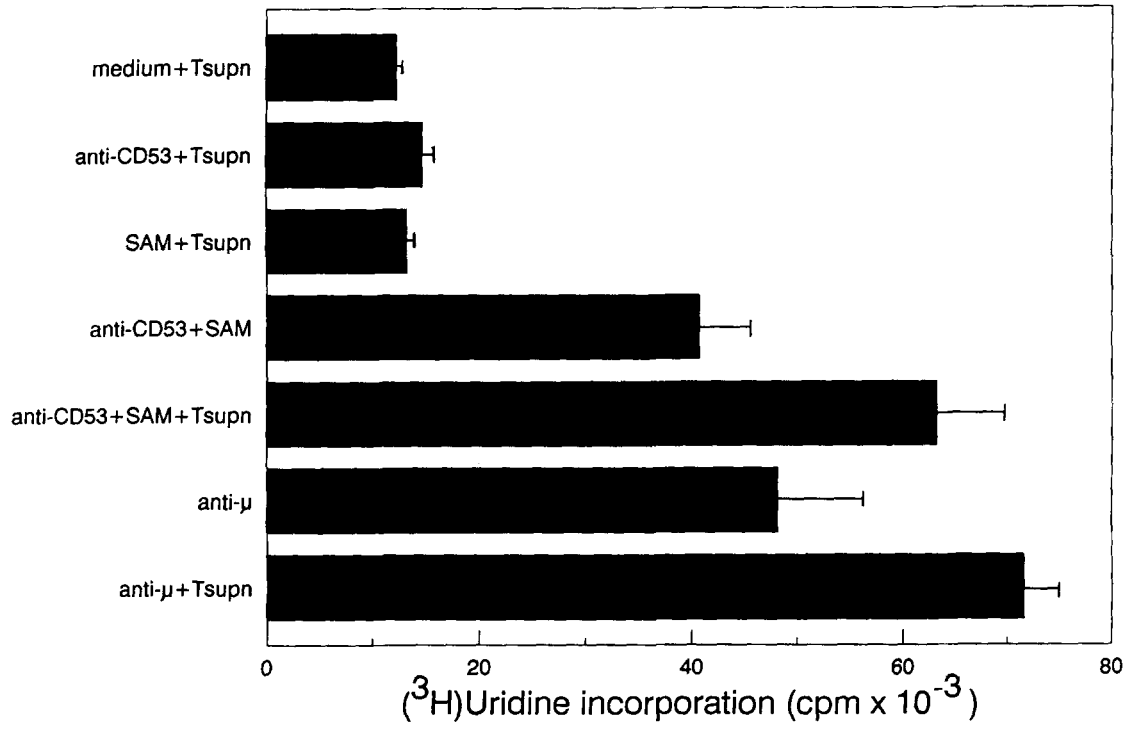


**FIGURE 3.** Cross-linking of the CD53 Ag induces RNA synthesis in the presence or absence of T cell supernatant. B cells were stimulated with anti-CD53 cross-linked with SAM or with anti-μ as described in *Materials and Methods*. RNA synthesis was determined by [$^3$H]uridine incorporation after 30 h of stimulation. Results are expressed as mean cpm ± SEM from three experiments.

activation (33). As shown in Figure 6, the increases in 7-AMD staining were similar in B cells stimulated for 48 h through cross-linking of the CD53 Ag and B cells stimulated with anti-μ, both in the presence of T cell supernatant (Fig. 6). As cells reach late $G_1$ phase, receptors for transferrin (CD71) appear, signifying the requirement of cells for transferrin-bound iron to initiate DNA synthesis (39). We used $CD69^-$ B cells to analyze the CD71 expression. Cross-linking of the CD53 Ag induced an increased CD71 expression after a 48-h stimulation (Fig. 7), although not to the same extent as did anti-μ (relative fluorescence intensity, 48 vs 24, respectively).

### *Effect of cross-linking of CD53 on cell cycle progression*

When B cells are stimulated in vitro, an increasing asynchrony occurs with time (37, 40). The first cells enter S phase 36 to 40 h after stimulation and, after 72 h, cells will be scattered through the first and second cell cycles. To examine cell cycle progression in detail, we performed multiparameter cell cycle analysis allowing estimation of cells in the first and second cell cycle as well as the distribution of cells within the different phases of each cell cycle after 48 and 72 h of stimulation (41, 42). The described staining method incorporates BrdU into DNA during DNA replication, resulting in quenching of the Hoechst 33258 staining. This makes it possible to distinguish cells from first and second cell cycles. Furthermore, by adding propidium iodide, the fluorescence of which is not affected by BrdU, it is possible to discriminate between different phases of each cycle. After 48 h less than 5% of cells had reached the second cell cycle in agreement with previous data (37 and data not shown). However, after 72 h, approximately 30% of B cells stimulated with anti-CD53 cross-linked with SAM or anti-μ, both in the presence of T cell supernatant, had traversed one cell cycle (Table I). When B cells were stimulated with anti-CD53/SAM in the absence of T cell supernatant, less than 2% of cells had reached the second cell cycle (data not shown). The cell cycle distribution data showed that in the presence of T cell supernatant only very few B cells stimulated with either anti-CD53/SAM or anti-μ passed beyond the $G_1$ phase in the second cell cycle after 72 h of incubation (Table I and Fig. 8). Approximately 28% of the B cells in the first cell cycle were in $S/G_2/M$ phases after stimulation with anti-μ compared with 15% of cells stimulated with anti-CD53/SAM (Table I and Fig. 8). Thus, cross-linking of anti-CD53 in the presence of T cell supernatant promotes slightly less efficient cell cycle progression as anti-μ/T cell supernatant. Taken together our data show that cross-linking of the CD53 Ag promotes efficient $G_1$ entry in the absence of T cell supernatant.

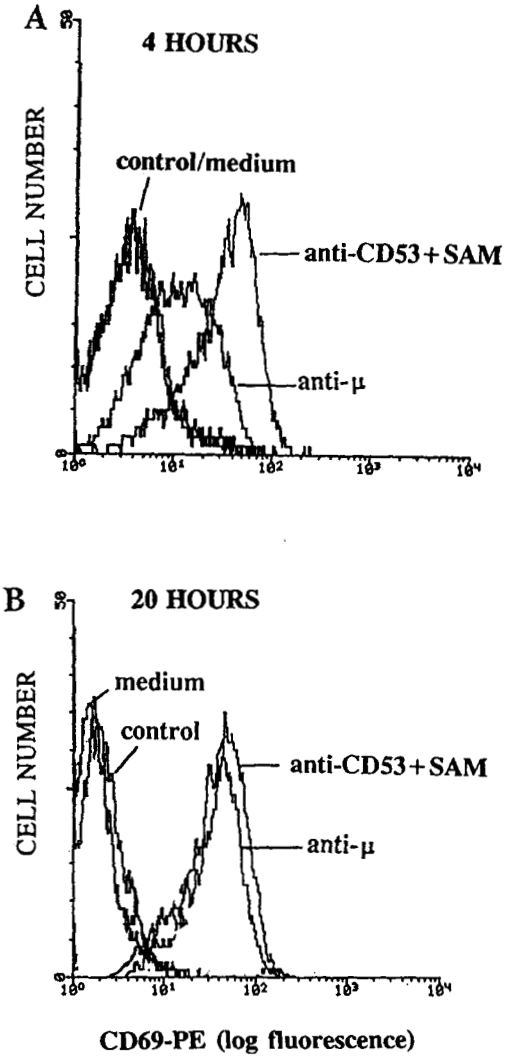


**FIGURE 4.** Effects of CD53 cross-linking on the expression of the early activation Ag CD69. CD69$^-$ B cells were stimulated for 4 h (A) or 20 h (B) with anti-CD53/SAM or anti-$\mu$, as described in *Materials and Methods*. Cells were stained with biotinylated anti-CD69 and streptavidin-phycoerythrin and flow cytometry was performed on a FACScan. Controls represent CD69$^-$ B cells stimulated with the irrelevant isotype-matched mAb anti-AFP/SAM (control) or incubated without stimuli for the same time intervals (medium). Cells stained with biotinylated anti-AFP and strepavidin-phycoerythrin were similar to the other negative controls. One representative experiment of five is presented.

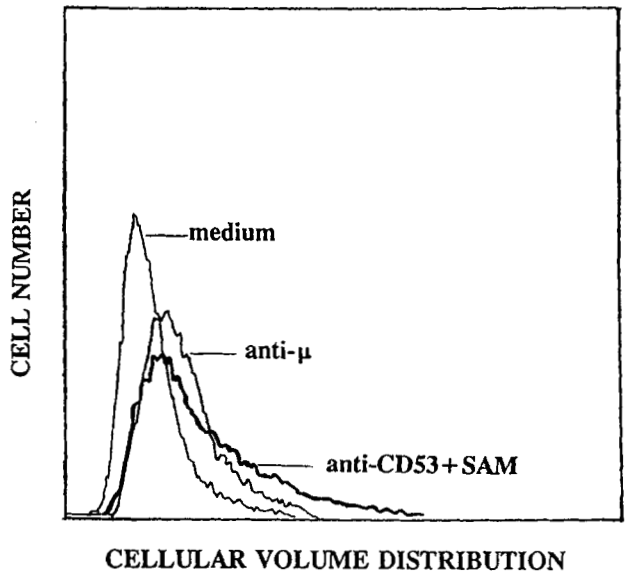


**FIGURE 5.** Effects of CD53 cross-linking on cellular volume distribution. B cells were stimulated for 20 h with anti-CD53/SAM or anti-$\mu$ as described in *Materials and Methods*. Unstimulated cells cultured in medium are shown for comparison. One representative experiment of four is presented.

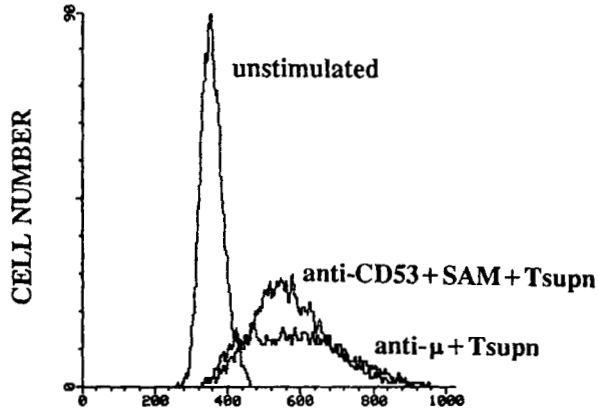


**FIGURE 6.** Effects of CD53 cross-linking on 7-AMD staining. B cells were stimulated for 48 h with anti-CD53/SAM or anti-$\mu$, both in the presence of T cell supernatant. 7-AMD staining was performed as described in *Materials and Methods* and was measured on a FACScan. Control cells (unstimulated) were cultured in medium alone. One representative experiment of three is presented.

### *Cross-linking of anti-CD53 on B cells induces IgG and IgM production in the presence of T cell supernatant*

We wished to investigate whether CD53 signaling also could induce Ig secretion. As shown in Table II, B cells stimulated through the CD53 Ag for 7 days in the presence of T cell supernatant secreted IgG and IgM. The level of Ig secretion induced by CD53 cross-linking in the presence of T cell supernatant was comparable with that of B cells stimulated with SAC in combination with T cell supernatant. Furthermore, B cells stimulated with anti-AFP/SAM did not induce IgM or IgG secretion (data not shown). B cells stimulated with anti-CD53 and SAM in the presence of IL-4 did not produce IgG or IgM (data not shown).

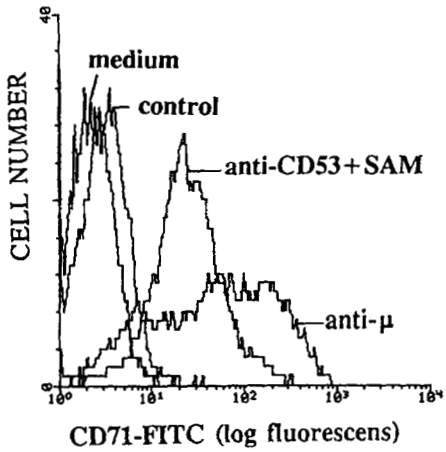


**FIGURE 7.** Effects of CD53 cross-linking on CD71 expression. CD69⁻ B cells were stimulated for 48 h with anti-CD53/SAM or anti-μ, as described in *Materials and Methods*. Cells were stained with anti-CD71-FITC and flow cytometry was performed on a FACScan. Controls represent CD69⁻ B cells stimulated with anti-AFP/SAM (control) or cells incubated without stimuli for 48 h (medium). Cells stained with an irrelevant IgG1-FITC were similar to the other negative controls. One representative experiment of five is presented.

## Discussion

In the present study we show that cross-linking of CD53, a member of the tetraspan family, induces polyclonal activation of B cells. Extensive cross-linking of the CD53 glycoprotein by means of a mAb and secondary Ab was necessary to achieve mid-to-late $G_1$ phase entry as measured by increased RNA synthesis, increase in CD69 and CD71 expression, enhanced 7-AMD staining, and increased cellular volume. Moreover, although few cells entered S phase by cross-linking of anti-CD53 alone, efficient proliferation and differentiation was observed in the presence of T cell supernatant or recombinant cytokines.

In addition to sIg, several other B cell surface molecules can mediate cell activation upon cross-linking. Some of them, such as CD19, CD21, and CD22 (43–46) need to be co-ligated with sIg to induce activation whereas others, such as CD20, CD40, CD72, and CD73 (47–50), and also CD53 as described here, exert the activation effects upon cross-linking independent of sIg. Cross-linking of CD53 causes early activation effects similar to those elicited through sIg. In addition to the activation characteristics reported in the present study, signaling through CD53 has recently been shown to induce a transient increase in cytoplasmic calcium concentration probably dependent on tyrosine kinase activity (26). However, in contrast to activation via sIg in which extensive cross-linking by means of anti-μ alone promotes S-phase entry, signaling through CD53 requires exogenous cytokines (IL-2 or IL-4) to progress from $G_1$ to S phase.

There is much evidence to support a two-step model for induction of B cell proliferation (51). Competence-inducing factors act on resting cells and promote $G_1$ entry and increased responsiveness to progression factors that act in $G_1$ and promote $G_1$-S transition. One important feature of complete induction is the increased expression of receptors for cytokines acting in $G_1$ (52, 53). Anti-μ has been shown not only to act as a competence signal, but also at high doses to act on $G_1$ cells and to promote $G_1$-S transition (37, 51, 54). In contrast, cross-linking of the CD53 Ag cannot induce progression beyond a restriction point in late $G_1$, but responsiveness to progression factors is induced, making the cells susceptible to exogenous factors such as IL-2 and IL-4. Taken together, the data indicate that cell activation through CD53 resembles activation by polyclonal anti-μ, except for the requirement for cytokines to pass a restriction point in late $G_1$. In the presence of T cell supernatant, the percentage of cells in the second cell cycle were similar in anti-μ and anti-CD53/SAM-treated cells after 72 h of stimulation. However, the observation that fewer cells within the first cell cycle had reached $S/G_2/M$ in cells stimulated with anti-CD53/SAM/T cell supernatant compared with cells stimulated with anti-μ/T cell supernatant suggests that some differences exist between these stimuli under optimal conditions with regard to passages through restriction points in the first cell cycle.

Table I. *The effect of CD53 cross-linking on cell cycle distribution*[a]

| Stimulus | % Cells | | Distribution of Cells | | | |
|---|---|---|---|---|---|---|
| | | | First cell cycle (%) | | Second cell cycle (%) | |
| | First cell cycle | Second cell cycle | $G_0/G_1$ | $S/G_2/M$ | G1 | $S/G_2/M$ |
| Anti-CD53 + SAM + Tsupn[b] | 68 ± 2 | 32 ± 2 | 85 ± 1 | 15 ± 1 | 97 ± 1 | 3 ± 1 |
| Anti-μ + Tsupn | 71 ± 2 | 29 ± 2 | 72 ± 2 | 28 ± 2 | 97 ± 1 | 3 ± 1 |
| SAC + anti-μ | 58 ± 4 | 42 ± 4 | 65 ± 2 | 35 ± 2 | 97 ± 1 | 3 ± 1 |
| Tsupn | 99 ± 1 | <1 | 99 ± 1 | 1 ± 1 | <1 | <1 |
| Medium | 99 ± 1 | <1 | 98 ± 1 | 2 ± 1 | <1 | <1 |

[a] B cells were stimulated for 72 h in the presence of the indicated stimuli. BrdU was added after 24 h. Cells were fixed in methanol and stained with Hoechst 33258 and propidium iodide. The percentage of cells in the second cycle was determined as the fraction of cells with low Hoechst 33258 fluorescence (see Fig. 8). The cell cycle distribution of cells in the first and second cycle were determined from the propidium iodide fluorescence distributions after gating on cells with high and low Hoechst 33258 fluorescence, respectively (see *Materials and Methods*). Results are expressed as mean ± SEM of six experiments from different donors.
[b] Tsupn, T cell supernatant.

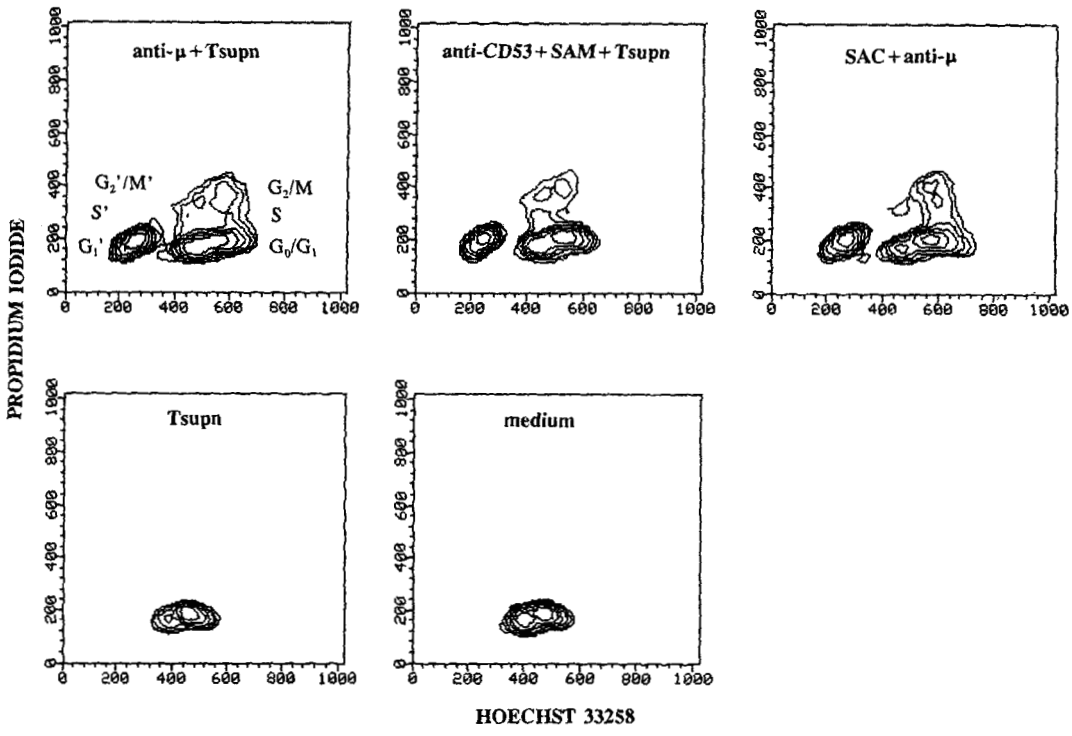


**FIGURE 8.** The effect of CD53 cross-linking on cell cycle distribution. B cells were stimulated as described in the legend to Table I. BrdU was added after 24 h of stimulation and the cells were fixed in methanol and stained with Hoechst 33258 and propidium iodide before analyzing cells with a Becton Dickinson FACStar$^{Plus}$ as described in *Materials and Methods*. $G_0/G_1$, S, and $G_2/M$ symbolize the cells in the first cell cycle, whereas $G_1'$, S', and $G_2/M'$ represent cells in the second cell cycle. One representative experiment of six is presented.

Table II. *Effect of CD53 cross-linking on Ig secretion*[a]

| Stimulus | IgM (ng/ml) | IgG (ng/ml) |
|---|---|---|
| Medium + Tsupn | 85 ± 20 | 86 ± 19 |
| SAC + Tsupn | 212 ± 38 | 269 ± 59 |
| Anti-CD53 + Sam + Tsupn | 181 ± 29 | 233 ± 60 |
| SAM + Tsupn | 71 ± 23 | 109 ± 19 |

[a] Purified B cells were stimulated for 6 days with the indicated stimuli before cells were washed and resuspended in RPMI 1640, 5% FCS, and T cell supernatant (Tsupn). After 24 h incubation, supernatants were harvested and the levels of IgM and IgG secretion were measured by ELISA as described in *Materials and Methods*. Results are expressed as mean ± SEM from five different experiments.

Thus, although it appears that anti-CD53-activated cells enter S phase less efficiently than anti-μ-stimulated cells, both in the presence of T cell supernatant, they proceed relatively more efficiently once they have passed the restriction point in the first $G_1$.

At this time it can only be speculated what may be the molecular basis for the ability of the CD53 molecule to mediate B cell activation. First, CD53 could simply be a receptor for some as yet unidentified soluble or cell-surface-bound ligand involved in a sIg-independent B cell activation. On the other hand, the predicted structure of the CD53 polypeptide chain, which apparently crosses the membrane four times, makes it an attractive candidate for a component of a membrane channel. For example, such a hypothetical calcium channel could be linked to sIg or some other receptor and be regulated by signaling through this. This hypothesis may be at least indirectly supported by the fact that several other topologically similar membrane proteins are components of ion channels. Thus, oligomeric structures composed of CD20 and possibly also other components are functional calcium channels (55). The best known analogous system is the large family of ligand-gated neurotransmitter receptors, composed usually of similar subunits, all of them spanning the membrane four times (56). However, CD53 has recently been found to be part of a multicomponent complex in the membrane of B cells, containing also three other tetraspan molecules (CD37, CD81 (TAPA-1), and CD82 (R2)), HLA-DR, and possibly other molecules such as CD19 and CD21 (57). These complexes are probably closely related if not identical to those described earlier containing CD19, CD21, and TAPA-1 (CD81) (58) or TAPA-1 and DR (59). A possible functional and structural relationship between these tetraspan-DR complexes to sIg was not examined, but it has been suggested that they might play a role in signaling through MHC class II molecules or through the CD19/CD21 complex. Interestingly, CD19 has been demonstrated to be physically linked to sIg (43). Several other interesting associations of the tetraspan molecules with

other membrane molecules have been described, such as CD81 (TAPA-1) and CD82 (C3/R2) with CD4 and CD8 in human T cells (60), CD53 (OX44) with CD2 in rat-activated NK and T cells (24), and CD63 with IgE receptor in rat basophils (61). It may therefore be hypothesized that CD53, as well as other tetraspan molecules, may be associated with various receptor molecules and possibly play some accessory role in cell activation. However, at this moment there are no direct indications that CD53 may be a component of a sIg-regulated ion channel, or some other sIg-linked accessory signaling structure. Furthermore, co-ligation of anti-$\mu$ and anti-CD53 did not lead to synergy even when both mAbs were used under suboptimal concentrations (data not shown). The lack of synergy between anti-$\mu$ and anti-CD53-mediated stimulation might indicate that CD53 does not function as a co-receptor for sIg capable of lowering the threshold of stimulus required to activate the cell.

Although mAbs against several members of the complexes on B cells, such as CD19, CD37, TAPA-1, and HLA-DR, have been found to induce growth inhibitory effects in some cells or to modulate sIg-mediated signaling after co-ligation, CD53 is the only member of these complexes that can induce B cell activation without co-ligation with sIgM (62–66). However, it should be noted that the functional effects of mAbs may be profoundly dependent on their epitope specificity. One might imagine that perturbation of an epitope in a receptor-regulated ion channel may cause its protracted opening, whereas ligation of another epitope may lead to its permanent closing, resulting in exactly opposite functional outcomes. This is in fact the case for different mAbs reactive with the CD20 Ag (67–69). The reason for such a discrepant result may also be mainly technical such as the use of different experimental systems (different types of cells, different activation and costimulatory conditions, soluble vs cross-linked Abs, etc.). The explanation will ultimately be provided by more detailed structural and functional studies. To elucidate these points, it would be desirable to test under identical conditions a panel of mAbs against various epitopes on different tetraspan molecules. However, whatever mechanism will prove correct, our data show that the CD53 Ag is an important component of a receptor system involved in B cell activation and differentiation.

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